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Deciphering the interplay between cysteine synthase and thiol cascade proteins in the survival of L. donovani under oxidative stress

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Background: *Leishmania* possess a unique trypanothione-dependent redox metabolism with pivotal role in protection from oxidative damage and drug resistance. The cascade of trypanothione biosynthesis depends on L-cysteine as the precursor, whereas, cysteine bioavailability is itself dependent on the cysteine biosynthesis pathway which includes enzyme cysteine synthase (CS). However, despite the apparent dependency of redox metabolism on cysteine biosynthesis pathway, the role of CS in drug resistance and redox homeostasis has remained unexplored. Herein, we have attempted to investigate the role of LdCS in Amphotericin B (Amp B) sensitive vs. resistant isolates of *L. donovani*.

Methods & Materials: LdCS was cloned in pXG-GFP^{+} vector to express LdCS as fusion proteins with a C-terminal GFP tag. The construct LdCS-GFP was transfected by electroporation in the *L. donovani* sensitive strain promastigotes and transformants selected upto final concentration of 200 μg/ml G418. MTT assay was performed to determine IC_{50} value of LdCS-GFP overexpressor and Amp B sensitive strains of *L. donovani* under different ROS inducers such as, H_2O_2, menadione and SNAP. Further, ROS levels, thiol content and enzymatic activities of LdCS, peroxidase and SOD were analyzed.

Results: Our results demonstrate stage-specific increase of LdCS expression and its enzymatic activity, accompanied by a higher thiol content, which implies that LdCS is upregulated in Amp B resistant isolates and during stationary stages of growth to meet the increased thiol demand of respective stages/isolates culminating into enhanced stress tolerance. In fact, overexpression of LdCS-GFP in sensitive strains imparted enhanced oxidative stress tolerance to the over-expressing parasites as compared to the wild type (WT) parasites. The IC_{50} values of LdCS-GFP toward H_2O_2, menadione and SNAP was found to be 217 ± 8.7 μM, 16.5 ± 2.5 μM and 370 ± 9.7 μM, respectively, which was ~1.87, ~2.21 and ~1.34 fold higher than WT parasites. Furthermore, enzymatic assays, thiol content and immunoblot analysis showed that these oxidants induced LdCS-GFP, as well as endogenous CS and thiol cascade proteins expression in *L. donovani* suggesting a ROS regulated mechanism of LdCS expression and thiol pathway proteins.

Conclusion: The LdCS expression is modulated by ROS probably to cater the metabolic demands of trypanothione and hence, alleviate oxidative stress.

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Investigating changes in monocyte phenotypes and functions in active visceral leishmaniasis patients

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Background: Visceral Leishmaniasis (VL) is a major public health problem in the Indian subcontinent. Mononuclear cells have direct role in initiation of inflammation, anti parasitic defences and ultimately in maintenance of tissue homeostasis. Monocytes play an important role as immune effector cells but their role in VL is not well known. We hypothesised that M2 macrophages play an important role in VL pathogenesis and aimed to characterise monocytes phenotypically and functionally.

Methods & Materials: We established M1 and M2 macrophage polarization dynamics in the whole blood of active VL patients at an intervals of 7 days for four weeks until their drug treatment was complete. Monocytes were phenotypically charecterised by immunophenotyping studies for chemokine receptors CCR2, CX3CR1, CCR7 and cell adhesion molecules VCAM1, ICAM1, PECAM1. Functional charecterization includes quantitation of intracellular TNF-α, IL-6, IL-1β production, gene expression level measurement of various M1, M2 markers in CD14 enriched cells from active and cured VL cases and in endemic healthy controls. Myeloperoxidase expression, phagocytosis and intracellular *Leishmania* parasite killing by monocytes were also examined.

Results: We found only minor changes in the frequency of M1 and M2 macrophages at the beginning and end of drug treatment, but observed several significant changes in the expression of cell surface markers (CD206, CD163) used to identify these cell subsets. In particular, we found that M2 macrophages increased in frequency and changed expression of cell surface molecules 14 days after drug treatment commenced, suggesting a potential role in tissue repair and homeostasis. We also observed significantly upregulated expression of several M2 markers (TGM2, PKM, SLC2A1) at gene level. We found reduced expression of CD14 on monocytes from active VL patients, but exacerbated TNF-α, IL-6 and dercreased production of IL-1β in response to SLA/LPS stimulation, compared with the same cells from drug cured and endemic control samples. We also found decreased myeloperoxidase expression by monocytes in VL.

Conclusion: Together, our findings indicate dynamic changes to macrophage and monocytes populations in VL patients over the course of drug treatment, and suggest that the functions of these cells may change at different stages of disease. We found upregulation of some markers for monocyte deactivation.

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Microplate whole blood interferon-γ release assay for marker of Leishmania donovani infection

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Background: Laboratory tests which can be produced in a reproducible and scalable manner are much needed to identify visceral leishmaniasis (VL) infection and fulfill the goals of the elimination campaign. Whole blood interferon gamma (IFN-γ) release assay (IGRAs) is an *in vitro* immune test that has recently been developed as an alternative to the Leishmanin skin test (LST) for identification of individuals exposed to *L.donovani* infection but without disease. Requirement of 3 ml of blood preclude this test widely acceptable for larger community based studies. This study aimed at evaluating the performance of microplate based IGRA using 300 μl blood (direct blood as well as 1:1RPMI diluted blood) with conventional IGRA (3ml blood) to establish this assay as an epidemiological tool for marker of infection.

Methods & Materials: We employed conventional IGRA and microplate based IGRA with direct as well as diluted venous blood using soluble leishmania antigen (SLA) on patients with active visceral leishmaniasis (VL, n = 32), patients with cured VL (n = 20), endemic Healthy Controls (EHC, n = 21) and healthy control subjects living in non-endemic area (NEHC, n = 12). IFNγ levels in culture supernatants were measured by ELISA and kappa statistics was used to access the concordance between test assay formats.

Results: The whole blood cells of both active VL and cured VL produced significantly level of IFN-γ in both format of IGRA. Positive correlations were found with active VL blood in IFN-γ production between 3 ml vs 600 μl, 3 ml vs 300 μl, and 600 μl vs 300 μl, while with cured VL blood it was moderate with 3 ml vs 600 μl; 3 ml vs 300 μl. No significant difference in the overall IFN-γ response by both assay formats was detected, and agreement between tests was significant.

Conclusion: We demonstrate a reliable and scalable process similar in sensitivity to conventional IGRA, but with the advantage of 10 times less venous blood requirement and higher through-put. Development of microplate based IGRA format will be useful tool for providing the means to more efficient screening in large scale immunological and epidemiological studies and fill an unmet need in the VL elimination campaign on the Indian sub-continent.

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Decreased miltefosine susceptibility in clinical isolates of Leishmania donovani derived from visceral leishmaniasis and post kala-azar dermal leishmaniasis: Apparent mechanisms and clinical implications

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Background: Miltefosine is an oral antileishmanial drug. Recent reports indicate a significant decline in its efficacy and high relapse rate in visceral leishmaniasis (VL) and post kala-azar dermal leishmaniasis (PKDL). Miltefosine susceptibility of relapse case isolates were >3 fold lower compared to pre treatment isolates.

Methods & Materials: To understand mechanism responsible for miltefosine unresponsiveness, we determined (i) the sequence of LdMT and LdRos genes implicated in miltefosine translocation (ii) miltefosine accumulation and reactive oxygen species (ROS) tolerance and (iii) transcriptome profile in clinical isolates of Leishmania donovani obtained from VL and PKDL patient at pre treatment and clinically relapsed stages as well as miltefosine resistant parasite(LdM30).

Results: LdMT gene sequencing revealed the previously reported single-nucleotide polymorphism, C1259→A resulting in substitution of Thr 420→Asn and a novel SNP T527-A resulting in substitution of Val176-Asp resistant parasites. L. donovani parasites from VL and PKDL patients relapsed after miltefosine treatment exhibited significantly lower accumulation of miltefosine compared with wild type parasites. Miltefosine induced ROS levels were significantly low ($p<0.05$) in macrophages infected with LdM30 and parasites from relapse cases compared to wild type parasites, indicating better tolerance for oxidative stress in unresponsive clinical isolates. Transcriptome profiling revealed that several genes involved in antioxidant defense mechanism (TRYP, Cyt b5 Red, TSH), metabolic process (Lipase precursor, PGMPUT), transporters (VPTM, MDRP), cell component and cell motility (SMP2, NUP155, CYP) are preferentially expressed in LdM30 and relapsed case parasites than wild type L. donovani parasites. Several other genes mainly transporters like ABCF2, amino acid transporter, surface acylated putative protein, APH and mitochondrial precursor peptide, chaperon TCP20, clathrin coated assembly protein, C5 sterol desaturase, autophagy protein ATG10 were preferentially expressed in wild type parasite compared to relapse case and LdM30 parasites.

Conclusion: The present study provides the understanding of parasitic factors and pathways responsible for miltefosine unresponsiveness in VL and PKDL. Decreased miltefosine susceptibility and increasing relapse rate in VL and PKDL patients indicate the declining efficacy of monotherapy with miltefosine and warrants the need of introducing alternate drugs/ combination therapy with miltefosine.

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